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13. ABSTRACT *(Maximum 200 Words)*

Mammary gland (MG) stem cells, like stem cells in other tissues, are quiescent cells that retain an enormous capacity for self-renewal and differentiation throughout many years of life. These primitive cells are thought to be a likely target for tumorigenesis, possibly representing the origin of some types of breast cancer.

In this study, we identified and purified MG stem cells in order to study their role in the development and therapy of breast cancer. A method to previously developed purify stem cells from murine bone marrow war initially employed. Stem cells specifically expel the fluorescent dye Hoechst 33342. Bone marrow and muscle cells with low dye fluorescence, as judged by flow cytometry (side population or "SP" cells), are extremely active and homogeneous stem cells. Staining of MG cells with Hoechst dye revealed a similar dye effluxing population. We hypothesized, therefore, that such MG-SP cells contain an enriched population of mammary epithelial stem cells. The original objectives of this award were to: 1) compare the physical characteristics of MG-SP cells, including morphology and size, with those of previously recognized MG stem cells; 2) determine if cell surface markers and cell cycle status of MG-SP cells are consistent with their stem cell candidacy; and 3) determine if MG-SP cells function as MG stem cells in murine transplantation experiments.

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INTRODUCTION:

The cellular origin of breast cancer remains elusive, impeding efforts to develop more effective therapies and better prognostic methods. Transplantation experiments indicate that ductal morphogenesis and lobuloalveolar development are driven by mammary gland (MG) stem cells, which, like stem cells in other tissues, are quiescent cells that retain an enormous capacity for self-renewal and differentiation throughout many years of life. These primitive cells are thought to be a likely target for tumorigenesis, possibly representing the origin of some types of breast cancer.

In this study, we proposed to identify and purify MG stem cells in order to study their role in the development and therapy of breast cancer. We developed previously a method to purify stem cells from murine bone marrow, and demonstrated its utility for skeletal muscle stem cell purification. Stem cells specifically expel the fluorescent dye Hoechst 33342. Bone marrow and muscle cells with low dye fluorescence, as judged by flow cytometry (side population or "SP" cells), are extremely active and homogeneous stem cells. Staining of MG cells with Hoechst dye revealed a similar dye effluxing population. We hypothesized, therefore, that such MG-SP cells contain an enriched population of mammary epithelial stem cells. The original objectives of this award were to: 1) compare the physical characteristics of MG-SP cells, including morphology and size, with those of previously recognized MG stem cells; 2) determine if cell surface markers and cell cycle status of MG-SP cells are consistent with their stem cell candidacy; and 3) determine if MG-SP cells function as MG stem cells in murine transplantation experiments.

BODY:

Mammary epithelium can functionally regenerate upon transplantation. This renewal capacity has been classically ascribed to the function of a multipotent mammary gland stem cell population which has been hypothesized to be a primary target in the etiology of breast cancer. Several complementary approaches were employed in this study to identify and enrich mammary epithelial cells that retain stem cell characteristics. Using long-term BrdU labeling, a population of label retaining cells (LRCs) that lack expression of differentiation markers have been identified. LRCs isolated from mammary primary cultures were enriched for stem cell antigen-1 (Sca-1) and Hoechst dye-effluxing "side population" properties. Sca-1^{pos} cells in the mammary gland were localized to the luminal epithelia using Sca-$1^{+/GFP}$ mice and were progesterone receptor negative. Finally, the Sca-1^{pos} population was enriched for functional stem/progenitor cells, as demonstrated by its increased regenerative potential when transplanted into the cleared mammary fat pads of host mice. These studies are described in detail in the appended publication entitled, "Sca-1^{pos} Cells in the Mouse Mammary Gland Have Increased Regenerative Potential and Represent a Stem/Progenitor Cell Population", which is being considered for publication.

KEY RESEARCH ACCOMPLISHMENTS:

- Characterized a population of BrdU label retention cells in the mouse mammary gland that exhibit stem cell properties.
- Characterized the properties of mammary gland SP cells.

- Identified Sca1 as a stem/progenitor cell antigen in the mammary gland and enriched for Sca1 positive cells with increased regenerative potential in reconstituted mammary gland transplantation experiments.
- This represents the first isolation of a potential stem cell population in the mammary gland.

REPORTABLE OUTCOMES:

One appended manuscript has been submitted for publication.

CONCLUSIONS:

This study represents the first isolation of a potential stem/progenitor cell population in the mammary gland. Additional markers need to be identified to further subdivide this population potentially into the most primitive pluripotent stem cells and committed mammary gland progenitors. This will be accomplished by the analysis of other markers, such as integrins and cell surface glycoproteins, as well as the use of gene arrays on the isolated SP and Sca1-enriched populations. Additional transplantation studies are required to demonstrate that the Sca1-enriched populations exhibit self-renewal following transplantation. Finally, studies of the senescence properties of these cells need to be performed. Following the identification of additional markers and the characterization of these cells, it will be of interest to determine their relevance in breast cancer.

APPENDICES: copy of paper from *Welm et al.*.

Sca-1^{pos} Cells in the Mouse Mammary Gland Have Increased Regenerative Potential and Represent a Stem/Progenitor Cell Population

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Abstract

Mammary epithelium can functionally regenerate upon transplantation. This renewal capacity has been classically ascribed to the function of a multipotent mammary gland stem cell population, which has been hypothesized to be a primary target in the etiology of breast cancer. Several complementary approaches were employed in this study to identify and enrich mammary epithelial cells that retain stem cell characteristics. Using long-term BrdU labeling, a population of label retaining cells (LRCs) that lack expression of differentiation markers have been identified. LRCs isolated from mammary primary cultures were enriched for stem cell antigen-1 (Sca-1) and Hoechst dye-effluxing "side population" properties. Sca-1^{pos} cells in the mammary gland were localized to the luminal epithelia using Sca-$1^{+/GFP}$ mice and were progesterone receptor negative. Finally, the Sca-1^{pos} population is enriched for functional stem/progenitor cells, as demonstrated by its increased regenerative potential when transplanted into the cleared mammary fat pads of host mice.

Introduction

A unique characteristic of the mammary gland is its ability to proliferate, terminally differentiate and involute upon successive cycles of pregnancy, lactation and involution, respectively. Mammary epithelium is also capable of completely and functionally regenerating upon transplantation. This impressive renewal capacity has been classically ascribed to the function of a multipotent mammary gland stem cell population (For review see (Smith and Chepko 2001)).

The existence of mammary gland stem cells was first demonstrated through classical transplantation studies conducted by DeOme (DeOme et al. 1959). DeOme and colleagues observed that epithelium isolated from several different regions of the mammary gland at any stage of postnatal development was capable of generating functional mammary epithelial outgrowths containing ductal, lobuloalveolar and myoepithelial cells. Subsequent studies by Daniels indicated that mammary epithelium could be serially transplanted, but exhibited senescence following seven or eight transplants (Daniel et al. 1968). Kordon and Smith suggested, through the use of retroviral-tagging, that progeny from a single cell can give rise to a complete mammary gland upon transplantation (Kordon and Smith 1998). These data indicate that mammary gland stem cells are distributed throughout the gland, and exhibit a potent yet limited self-renewal capacity.

A stem cell population would be expected to contribute to the cell types in mammary gland development through symmetric cell division (for self-renewal) and asymmetric cell division (to generate progenitors) (Chepko and Smith 1997). During ductal morphogenesis (3-8 weeks of age in the mouse), the mammary gland is rapidly proliferating and contributing cell lineages to the developing epithelium. At the terminal ends of the ducts are bulb-like structures

called terminal end buds (TEB). TEBs are the proliferating units of the mammary gland, and as they penetrate the fat pad, their progeny make up the luminal and myoepithelial cell types of the gland. At the distal tip of the TEB exists a layer of highly proliferative cells termed "cap cells" that lack steroid receptors and are thought to represent an undifferentiated, stem/progenitor cell population, giving rise to differentiated luminal and myoepithelial cells in the subtending ducts (Williams and Daniel 1983; Daniel and Silberstein 1987). In the mature animal, the mammary gland remains in a quiescent state until the onset of pregnancy. During this period, stem cells are thought to be scattered throughout the gland and contribute to the maintenance of the ductal epithelial network.

Several morphologically distinct cell populations have been identified in the mammary gland. These cell types are distinguished based on DNA condensation, size, shape, location, cytoplasmic granularity, and nuclear morphology using electron microscopy. These distinctions have been used to detect a population of cells termed "small light cells" (SLC), which make up about 3% of the total epithelial component in rat mammary glands. SLCs exhibit morphological characteristics of putative undifferentiated, division-competent mammary gland stem cells (Chepko and Smith 1997; Smith and Chepko 2001). Although the morphological characteristics of putative mammary gland stem cells and their distribution throughout the gland during postnatal development have been described, their purification and functional analysis has not yet been accomplished.

Mammary gland stem cells have been hypothesized to be the primary targets in the etiology of breast cancer, and have been implicated as targets for carcinogenesis in several model systems. The mitotic and self-renewal capacity of stem cells are thought to make them highly susceptible to mutagenesis. In the rat, putative stem cells (termed "intermediate cells") are

thought to be the target of the chemical carcinogens dimethylbenz(a)anthracene (DMBA) and N-methylnitrosourea (NMU) (Russo et al. 1982; Russo et al. 1983). Primary tumors arising from carcinogen treatment express markers for intermediate cells as well as luminal, myoepithelial and alveolar cell types. In addition, carcinogen treatment during ductal morphogenesis (pubescent growth), a time when stem cells are thought to be highly proliferative, dramatically increases the onset of tumorigenesis (Russo et al. 1982; Russo et al. 1983; Russo and Russo 1996). In humans it was shown that the most susceptible population to develop breast cancer in the aftermath of the Hiroshima and Nagasaki atomic bomb explosions were pubescent and prepubescent females (McGregor et al. 1977). It is during this stage when TEBs are found in their highest numbers in the human breast (Dawson 1934; Russo and Russo 1987). Thus, the identification and isolation of mammary gland stem cells should both help elucidate important aspects of normal mammary gland development, as well as contribute to our understanding of the etiology of breast cancer.

This study describes for the first time the enrichment of a cell population within primary mouse mammary epithelial cell (MEC) cultures that displays multipotent mammary gland stem cell characteristics. BrdU label-retention, FACS analysis, and transplantation methods have been combined to enrich and characterize these cells and to determine their functional capacity for regeneration.

Results

BrdU Label Retention in Mammary Epithelial Cells

In an effort to identify quiescent stem cells in the mammary gland, a bromodeoxyuridine (BrdU) label-retention approach was employed that has been used successfully to identify stem cells in the skin (Lavker and Sun 1982), cornea (Cotsarelis et al. 1989), and hair follicle (Cotsarelis et al. 1990; Taylor et al. 2000). BrdU was administered continuously to mice undergoing ductal morphogenesis when stem cells are proliferating and should, therefore, incorporate the thymidine analog into their DNA. The labeling period was followed by a 9-week chase period during which BrdU retention was monitored via immunofluorescence using a FITC-conjugated anti-BrdU antibody. During the chase, ductal proliferation will continue until the ducts reach the edge of the mammary fat pad. At the end of ductal morphogenesis, the mammary gland will remain relatively quiescent, and stem cells are expected to exhibit lower proliferative and apoptotic indices than progenitor and more differentiated cell types. Under these conditions, quiescent stem cells should retain BrdU while the proliferating and terminally differentiated cell types will lose the label either following mitosis or after undergoing apoptosis.

Specifically, a continuous dose of BrdU was administered to 3-week old female mice for 14 days by means of an intrascapularly implanted Alzet minipump (Fig. 1A). After the 2-week BrdU dose, minipumps were removed and mammary gland biopsies were taken every week for nine weeks to monitor label-retaining cells. Approximately 70% of MECs were labeled during the 2-week dose of BrdU (Fig 1B,F). At the end of the 9-week chase period, less than 5% of luminal epithelial cells retained BrdU (Fig.1C,F), and these cells were termed label-retaining cells (LRCs). After the chase period, LRCs ranged in intensity from brightly labeled (C, arrows)

to dimly labeled (C, arrowheads), suggesting that LRCs represent a spectrum of cells that have undergone varying numbers of cell divisions.

To characterize the differentiation status of LRCs in the mammary gland, MECs were co-stained for expression of BrdU and either keratin (K)14/18 or the progesterone receptor (PR). After the 2-week dose with BrdU, approximately 40% of $BrdU^{pos}$ cells also expressed PR (Fig. 1D, F). Throughout the chase period the number of co-localizing cells steadily decreased to approximately 1.5% after nine weeks (Fig 1E,F). The total percentage of PR^{pos} cells did not significantly change during this time period, which is consistent with previous reports (Seagroves et al. 2000). A population of LRCs was detected which did not express K-14 or -18, markers of the myo- and luminal epithelium, respectively (Fig. 1G,H,I,J; arrows). Additionally, a sub-population of label-retaining luminal cells did express K14/18 (arrowheads), which correlates with a model in which label retaining cells represent a spectrum of quiescent stem cells and early differentiating cells (see Discussion). Thus, these data demonstrate that a population of LRCs do not express common mammary gland differentiation markers, suggesting they are maintained in a less differentiated state.

MG-SP Cells in the Mammary Gland

The efficient efflux of the fluorescent dye Hoechst-33342 has been demonstrated previously to be a mechanistic characteristic of pluripotent hematopoietic stem cells, (Goodell et al. 1996) . In those experiments, bone marrow cells were treated with Hoechst dye and then analyzed by FACS at two different emission wavelengths. A small, distinct population of bone marrow termed "side population" (SP) cells effluxes the Hoechst dye. Further, these SP cells contain the entire hematopoietic potential of whole bone marrow, establishing their functional

capacity as hematopoietic stem cells (Goodell et al. 1996). SP cells with multipotent stem cell characteristics also have been identified in other regenerative tissues, such as muscle (Gussoni et al. 1999; Jackson et al. 1999) and liver (G. Wulf, M. Goodell, submitted). Additionally, SP cells from bone marrow and muscle are enriched for the expression of stem cell antigen-1 (Sca-1), a cell surface protein shown to be a marker of pluripotent hematopoietic stem cells (Spangrude et al. 1988; Goodell et al. 1996; Jackson et al. 1999). A similar SP cell staining protocol was employed to isolate potential mammary gland stem cells from primary MEC cultures. Mammary glands were isolated from mice and manually and enzymatically digested as previously described (DeOme et al. 1959; Pullan and Streuli 1996). These cells were then stained with Hoechst dye and analyzed by FACS. Similar to bone marrow, the mammary gland contained a distinct population of Hoechst-effluxing SP cells, referred to as MG-SP cells (Fig. 2A). The MG-SP population represented approximately 2-3% of the total population of epithelial cells in the mammary gland, which is consistent with previous estimates of the percentage of stem cells in the mammary gland (Chepko and Smith 1997). Treatment with verapamil, a multi-drug transporter inhibitor shown to eliminate the SP population in bone marrow, also reduced the MG-SP population by four-fold (data not shown). Thus, the mammary gland contains a population of cells which efflux Hoechst dye in a manner similar to that described previously for hematopoietic and muscle stem cells.

Analysis of MG-SP Cells

Since Sca-1 is expressed in functional hematopoietic stem cells (SP cells), it was hypothesized that Sca-1 might also be expressed by the MG-SP cells. Primary MECs were incubated with Hoechst dye, stained with an anti-Sca-1 antibody, and then analyzed by FACS.

About 20% of the total population of the MECs and 75% of the MG-SP cells were found to be Sca-1^{pos} (Fig. 2B,C). These data suggest that the MG-SP population is enriched in Sca-1^{pos} cells.

To minimize the possibility of contamination from blood and lymphatic cells, only inguinal and pelvic mammary glands which were devoid of lymph nodes were used to make primary cultures. Expression of blood cell markers was analyzed in the MG-SP and Sca-1^{pos} populations to determine if these cells resulted from blood contamination. MG-SP cells did not express c-Kit or CD-45, markers of hematopoietic stem cells and peripheral blood (Fig 2C and data not shown). In addition, the Sca-1^{pos} population in total MEC culture was found to be largely CD-45 and lineage marker negative (Fig 2D and data not shown). Similar MG-SP and Sca-1^{pos} populations were also isolated from primary MECs after culturing cells for 5 days with daily media changes (data not shown). These data suggest that the MG-SP and Sca-1^{pos} populations isolated from mammary gland primary cultures were not derived from blood cell contamination.

Label Retention in SP Cells

To determine the proliferative nature of the MG-SP cells, the presence of LRCs was analyzed in this population. Primary MECs were isolated from mice that received the long BrdU label followed by the 9-week chase. These cells were then FACS sorted for MG-SP cells and $BrdU^{pos}$ cells were determined by immunofluorescence. A four-fold increase in LRCs in the MG-SP population was detected as compared to the non-SP population (Fig.2E). These data suggest that MG-SP cells proliferate during ductal morphogenesis, but in the mature gland they have reduced proliferation and turnover compared to non-MG-SP cells.

Outgrowth Potential of Purified MG-SP Cells

To determine if MG-SP cells are capable of repopulating the mammary gland, MG-SP cells were isolated and transplanted into cleared mammary fat pads in limiting numbers. In this experiment, MECs were isolated from C57BL6/129 ROSA26 (ROSA) donor mice and cultured for five days. Cells were cultured in this experiment in an effort to improve their viability through the SP-staining and sorting procedure. Cultured MECs retained a MG-SP profile similar to fresh MECs (data not shown). MG-SP cells from the ROSA primary cultures were then isolated by FACS and limiting numbers of cells were mixed with $2x10^5$ wild type C57BL6 primary MECs. This competitive repopulation protocol was used to ensure the presence of any paracrine interactions between MG-SP and non-MG-SP cells that may be required for proper outgrowth. The mixed cell populations were transplanted into the cleared fat pads of Rag-$1^{-/-}$ recipient mice. Immuno-compromised recipients were used to prevent host rejection of cells derived from the ROSA mixed background. Recipient mice were bred after six weeks to induce lobuloalveolar development in the mammary gland, and outgrowths were isolated two weeks later. Whole mounts of outgrowths were stained with X-gal to detect β-galactosidase (β-gal) activity, an indication that such cells originated from the ROSA MG-SP cells. Patches of β-gal-expressing cells could be detected in outgrowths from as few as $2.5x10^4$ transplanted ROSA MG-SP cells (data not shown), and robust staining was detected in outgrowths from $7.5x10^4$ transplanted ROSA MG-SP cells (Fig. 2F,G). In this whole mount, two distinct outgrowths were detected by X-gal staining (Fig. 2F, arrowheads). Higher magnification revealed β-gal-positive cells in both ductal (Fig.2G, arrowheads) as well as alveolar epithelium (Fig. 2G, arrows). These experiments indicate that purified MG-SP cells have mammary gland outgrowth potential and can contribute to alveolar and ductal epithelial populations. However, because of the loss of

viability of MG-SP cells following Hoechst staining and high pressure FACS sorting, it was not possible to obtain a precise estimate of the fold enrichment obtained in these experiments. Since the MG-SP population was found to be enriched in Sca-1^{pos} cells, Sca-1 expression in primary MECs was investigated as an alternative method of stem cell enrichment.

Localization of Sca-1^{pos} Cells in the Mammary Epithelium

The localization of Sca-1-expressing cells in the mammary gland was analyzed using a targeted GFP insertion into the Sca-1 locus (Sca-$1^{+/GFP}$) (T. Graubert et al, submitted). These mice are heterozygous for a mutation in which an EGFP cassette is inserted into the Sca-1 locus, placing it under the regulatory control of the Sca-1 promoter. Analysis of GFP fluorescence in the mammary gland revealed sporadic GFP expression scattered throughout the ductal luminal epithelium (Fig. 3A), an observation consistent with the theoretical expected distribution of stem or progenitor cells, as predicted from transplantation experiments. Additionally, GFP and PR expression observed in mature ducts did not co-localize in the same cells (Fig. 3B). Assuming PR expression is a measure of an increased differentiation state in the virgin mammary gland, these data suggest that the Sca-1^{pos} cells represent a population of less differentiated cells.

Sorting Mammary Epithelial Cells Based on Sca-1 Expression

The apparent relationship of MG-SP, LRCs and Sca-1^{pos} cells suggested that it might be possible to isolate mammary stem cells with improved viability using their Sca-1^{pos} status as a criterion for selection (Fig 4). Accordingly, an immunoaffinity technique was employed using an anti-Sca-1 antibody conjugated to microbeads via a biotin/streptavidin interaction. Freshly prepared primary MECs, isolated from inguinal mammary glands (to reduce muscle

contamination), were incubated with the biotinylated anti-Sca-1 antibody and streptavidin-conjugated beads. These were then applied to a column in a magnetic field. The Sca-1^{pos} cells that adhered to the magnetic column were washed and then eluted by removing the column from the magnetic field. FACS analysis verified that this method depleted Sca-1^{pos} cells in the flow-through fraction but enriched for a Sca-1^{pos} population in the eluate (Fig. 4A,B,C). The bound cell fraction (Fig. 4C) exhibited a 3.5-fold increase in Sca-1^{pos} cells, while the depleted population (Fig.4B) displayed a 3.5-fold decrease in Sca-1^{pos} cells, as compared to the starting MEC population (Fig.4A).

To determine directly if the mammary gland Sca-1^{pos} population contained a quiescent cell population capable of BrdU label retention, Sca-1^{pos} cells were purified using the label retaining protocol previously described. The percentage of BrdU label retention in Sca-1^{pos} population was determined by immunofluorescence. Approximately 19% of purified Sca-1^{pos} cells retained BrdU after the 9-week chase as compared to 7-8% of the cells in the Sca-1-depleted population (Fig 4D), a greater than two-fold enrichment. These data were confirmed by BrdU immunofluorescence and FACS analysis of Sca-1-enriched and Sca-1-depleted populations (data not shown). Thus, Sca-1^{pos} cells isolated from mouse mammary gland primary cultures contain an enriched population of growth quiescent cells, a characteristic consistent with their role as putative stem/progenitor cells.

Outgrowth Potential of Sca-1-Enriched Cells

The Sca-1 magnetic enrichment protocol generated a population of cells that displayed at least a 7-8 fold increase in the number of Sca-1^{pos} cells in the enriched population compared to the depleted population. To characterize the regenerative potential of Sca-1^{pos} cells,

transplantation experiments into cleared fat pads of syngeneic host mice were again employed using the Sca-1-enriched and Sca-1-depleted populations of freshly prepared primary MECs. Transplants were removed from mice after 6-8 weeks and the whole mounts were stained with hematoxylin to identify the epithelium and the extent of outgrowth. When $5x10^4$ Sca-1-enriched primary MECs were injected into the cleared fat pads, 6 out of 6 transplants grew out from a focal injection site, while only two out of the six Sca-1-depleted transplants formed an outgrowth (Fig. 5). Some of the Sca-1-depleted transplants produced only rudimentary epithelial structures (Fig. 5B, arrowhead), while most of the Sca-1^{pos} transplants formed fully developed, morphologically normal outgrowths (Fig. 5A).

A complementary approach was also taken, using FACS sorting of primary MECs isolated from Sca-$1^{+/GFP}$ mice. Using this FACS-based method, the Sca-1^{pos} population was enriched to >90% positive, while the depleted population contained <2% Sca-1^{pos} cells (data not shown). Less than 10,000 viable cells were injected into each host fat pad, and outgrowths were harvested after six weeks. All six injections of Sca-1^{pos} cells resulted in an outgrowth, while none of the six Sca-1^{neg} injection sites grew out (Fig. 5A,B,C). These data complement the Sca-1-bead sorting technique, offering a purer enrichment of Sca-1 and subsequent increased outgrowth potential. Thus, Sca-1^{pos} cells isolated from mammary gland primary cultures appear be enriched for a population of multipotent progenitor cells and contain increased outgrowth potential.

Sections of the wholemounted outgrowths were stained with hematoxylin and eosin (H&E) and analyzed by light microscopy to study the morphology of Sca-1-enriched outgrowths. Sca-1^{pos} outgrowths contained normal luminal and myoepithelial cells as well as TEBs with distinct cap and body cells (Fig. 5D, E, F), indicative of normal ductal morphogenesis (Williams

and Daniel 1983). Additionally, Sca-1^{pos} outgrowths formed normal alveolar buds during early pregnancy (Fig. 5D,F). These data suggest that Sca-1^{pos} cells isolated from primary MEC culture have the potential to differentiate into multiple mammary epithelial cell types.

Discussion

In this study, several complementary approaches have been employed to identify and enrich mammary epithelial cells that retain stem cell characteristics. Long term BrdU label retention studies, FACS sorting for an MG-SP population, and Sca-1 enrichment all use differing criteria to identify cohorts of mammary epithelial cells with overlapping stem cell characteristics. These populations of cells collectively represent a spectrum of undifferentiated and differentiated cells with unique molecular and regenerative characteristics.

The Mammary Gland Contains an Undifferentiated Quiescent Cell Population

Non-uniform expression of the steroid hormone receptors (PR, ER) in luminal cells suggests the existence of mixed populations of cell types in the mature duct (Seagroves et al. 2000) S. Grimm, J. Rosen in preparation). Previous studies have indicated that a paracrine relationship may exist between these PR^{pos} and neighboring proliferative, PR^{neg} cells (Brisken et al. 1998; Russo et al. 1999; Seagroves et al. 2000). Thus, proliferating cells, identified either by a 2 hr BrdU pulse or Ki67 immunohistochemistry, were PR negative. These studies have suggested that PR^{pos} MECs may exit the cell cycle, but the mechanism of growth arrest has not been determined. However, >40% of cells continuously labeled for two weeks with BrdU expressed PR. These data suggest that PR expressing cells do not undergo proliferation, yet cells that have undergone proliferation are capable of eventually expressing PR. Although the exact lineage of differentiation in the mammary gland is unknown and controversial, we propose that PR^{pos} cells can arise from a proliferating population, possibly through differentiation.

Interestingly, after a one week chase, the total $BrdU^{pos}$ cells decreased by 50%, but the number of $BrdU^{pos}/PR^{pos}$ cells did not decrease significantly. This dramatic reduction in $BrdU^{pos}$

cells could be a result of apoptosis or dilution of the BrdU through proliferation. However, since the apoptotic index is very low at this stage of development, it is unlikely that cell death is contributing to the $BrdU^{pos}$ cell loss (Humphreys et al. 1996). This suggests that $BrdU^{pos}$ cells continue to proliferate and lose BrdU through mitotic dilution. However, the lack of change in the number of $BrdU^{pos}/PR^{pos}$ cells one week after minipump removal suggests that once BrdU labeled cells express PR, they remain quiescent, possibly due to exit from the cell cycle. It will be interesting to elucidate the expression profile of cyclin kinase inhibitors within the PR^{pos} cell population in the normal mammary gland epithelium. The data obtained in this study support the hypothesis that proliferating progenitor cells can give rise to more differentiated cells which then exit the cell cycle and remain quiescent.

Since long term BrdU administration would be expected to label a mixed population of progenitor and stem cells, a 9-week chase protocol was employed to identify quiescent cells with low turn-over rates. During the 9-week chase period, the percentage of BrdU cells that express PR decreased approximately 15-fold. At the end of a 9-week chase period, >95% of cells that retained BrdU did not express PR. Since PR^{pos} cells have exited the cell cycle, the loss of these cells may be explained by a higher rate of apoptosis in the PR^{pos} cells compared to the LRCs. This is consistent with studies that suggest that mammary gland stem cells are capable of efficient cell division with reduced turn-over, as demonstrated through the observed regenerative capacity of a single stem cell (Kordon and Smith 1998; Smith and Chepko 2001).

To characterize the differentiation status of the LRC population, deconvolution microscopy was employed to determine the co-expression of PR and cytokeratins in BrdU labeled cells. Less than 5% of LRCs express PR, suggesting that this quiescent cell population remains less differentiated. In addition to lacking steroid hormone receptor expression,

approximately 50% of LRCs do not express K14/18, markers of myo- and luminal epithelium, respectively (Fig.1I and data not shown). These data suggest that the LRCs represent multiple populations, including cells at various stages of early differentiation.

Mammary Gland "Side Population" Cells Retain BrdU Label

To further characterize the LRCs, the label retention protocol was employed in combination with a FACS-based protocol used previously to isolate hematopoietic and muscle stem cells. Stem cells isolated by this method 1) efflux Hoechst dye via a multi-drug transporter that is sensitive to verapamil inhibition, 2) appear as a distinct "side population" during dual color FACS analysis, and 3) demonstrate regenerative potential upon transplantation (Goodell et al. 1996; Gussoni et al. 1999).

In the mammary gland, the "side population" observed was similar to that seen in muscle and liver (Gussoni et al. 1999; Jackson et al. 1999) G. Wulf, M. Goodell, submitted). MG-SP cells could be isolated to >90% purity from primary MECs that were either freshly prepared or cultured for five days (Fig. 2 and data not shown). Interestingly, MG-SP comprised about 3-5% of the total mammary gland population regardless of the developmental stage (Fig. 2 and data not shown). This result is similar to the percentage of morphologically identified small light cells (SLC) described by Chepko and Smith, which have also been estimated to constitute approximately 3% of the total population during all stages of mammary gland development. SLCs are small, mitotically competent cells that are morphologically undifferentiated (Chepko and Smith 1997). Similarly, purified MG-SP cells were smaller than non-SP cells and scattered less light suggesting that these cells have less cytoplasmic complexity (data not shown).

To determine the presence of LRCs in the MG-SP population, MG-SPs were isolated from mice following long-term BrdU labeling and a 9-week chase period. Isolated MG-SP cells contained approximately four-fold more $BrdU^{pos}$ cells (LRCs) than the non-SP population. These data demonstrate that MG-SP cells do proliferate during ductal morphogenesis, but remain either more quiescent or less apoptotic than the non-SP population in the mature mouse mammary gland, consistent with their candidacy as mammary gland stem cells. In addition, when MG-SP cells were transplanted, they contributed to ductal and alveolar outgrowths. Several characteristics of the MG-SP cell population support a stem/progenitor nature of these cells: 1) MG-SP cells comprise a constant 3-5% of the total epithelial population in virgin and pregnant mice; 2) they proliferate when the mammary gland is rapidly establishing the ductal network; 3) they remain quiescent in the mature mouse; and 4) when transplanted, their progeny can develop into ductal and alveolar cell types.

$Sca\text{-}1^{pos}$ Population Contains MG-SP and Label Retaining Cells

FACS sorting of primary MECs (in the absence of Hoechst dye) decreased their viability by greater than five-fold (data not shown). The combination of long term proteinase treatment during primary MEC isolation, Hoechst dye treatment, and high pressure cell sorting makes obtaining viable MG-SP cells by this approach problematic. To isolate viable cells for transplantation, an alternative non-FACS-based technique was employed that used the cell surface marker Sca-1 to select stem and progenitor cells.

Stem cell antigen-1 (Sca-1) is a GPI-anchored protein frequently utilized in murine hematopoietic stem cell enrichment strategies (Spangrude et al. 1988). It is encoded by Ly-6A/E, a member of the Ly-6 superfamily of highly homologous genes conserved from humans to

snake (Ploug and Ellis 1994; Mao et al. 1996). Sca-1 is expressed on some differentiated cell types, including in the kidney and brain, and on T cells where it has been implicated as a costimulatory molecule (Malek et al. 1986; van de Rijn et al. 1989; Miles et al. 1997; Stanford et al. 1997). Since Sca-1 is expressed on both bone marrow- and muscle-derived SP cells (Goodell et al. 1996; Gussoni et al. 1999; Jackson et al. 1999), it was hypothesized that Sca-1 would also be expressed on MG-SP cells.

While the overall Sca-1^{pos} population in the mature mammary gland was about 20% of total epithelial cells, approximately 75% of MG-SP cells expressed Sca-1. Sca-1^{pos} cells isolated from the mammary gland did not express markers of blood cell-lineages (Fig. 2 and data not shown), and Sca-1^{pos} cells were retained in the MEC population even after culturing for five days. These data, in addition to the direct Sca-1-GFP localization in luminal ductal epithelium (Fig. 3), verify that the isolated Sca-1^{pos} cells are epithelial in nature and are not derived from contaminating blood or lymphatic cells. Therefore, Sca-1 appeared to be an ideal marker with which to enrich a MEC population for viable stem/progenitor.

To determine the presence of LRCs in the Sca-1^{pos} population, primary MECs were isolated from mice following long term BrdU labeling and a 9-week chase period. These cells were sorted based on Sca-1 expression using the magnetic cell sorting technique. BrdU incorporation, detected and quantitated by manual counting and FACS analysis, revealed that LRCs were enriched about two-fold in the Sca-1^{pos} as compared to the Sca-1-depleted population. Therefore, the Sca-1^{pos} and the MG-SP populations each contain a subpopulation of quiescent cells (LRCs) with low turn-over rates. However, since the MG-SP cells contain a four-fold enrichment in LRCs while Sca-1^{pos} cells are only enriched two-fold, this suggests that the MG-SP cells are less proliferative than the Sca-1^{pos} cells. One possible model to explain these

results is that Sca-1 is expressed in a broader population of cells that contain subpopulations of both MG-SP cells and LRCs (Fig. 6A). This subpopulation of Sca-1^{pos}/MG-SP/LRC cells may represent a less proliferative, more primitive population of progenitor cells.

Sca-1 is a Marker of Less Differentiated Luminal Epithelial Cells

To determine the spatial localization of the Sca-1^{pos} cells in the mammary gland, Sca-$1^{+/GFP}$ mice were utilized. These mice contain a targeted insertion of EGFP into the Sca-1 locus and express GFP under endogenous Sca-1 regulatory elements. These mice express GFP in a temporal and spatial manner similar to that observed for Sca-1 in hematopoietic cell lineages, and although this mutation disrupts the endogenous Sca-1 allele, Sca-1 haploinsufficiency has no important consequences for hematopoiesis (T. Graubert et al, submitted). GFP positive cells were localized specifically to the luminal epithelial layer of the mammary gland as observed by confocal microscopy. These data were confirmed using Sca-1-lacZ transgenic mice that express the Lac-Z gene under a 2.0 kb regulatory element from the Sca-1 promoter (Miles et al. 1997) (data not shown). In addition, GFP positive cells in mature ducts did not express PR. This result is in agreement with the previous observation in which PR is not expressed in LRCs.

Using confocal microscopy, some of the GFP positive cells appeared to contact the lumen. Interestingly, previous EM studies have shown that the small light cells (SLC) have limited contacts with the lumen and are primarily basally located (Chepko and Smith 1997). Since Sca-1^{pos} cells consistently comprised about 20% of the total population of the mammary epithelium and exhibited contact with both the lumen and basement membrane, it is unlikely that these cells exclusively represent the SLC population. Rather, it is possible that the SLCs

comprise a subpopulation of the larger Sca-1^{pos} population; likely candidates for the SLC subpopulation are the LRC/MG-SP cells.

Mammary Epithelial Sca-1^{pos} Cells Have Increased Outgrowth Potential

Smith and colleagues have suggested by retroviral tagging experiments that the progeny from a single cell may have the capability of regenerating the entire mammary epithelium (Kordon and Smith 1998). These investigators have also demonstrated that at least three multipotent cell populations with distinct outgrowth potential exist in the mammary gland. These three multipotent cell populations include cells capable of 1) complete mammary gland outgrowth, 2) ductal morphogenesis, or 3) lobuloalveolar development (Chepko and Smith 1997). To elucidate the outgrowth potential of the Sca-1^{pos} cells and their ability to contribute progeny to ductal and lobuloalveolar cell types, Sca-1-enriched and -depleted cells were transplanted into host mice. Sca-1^{pos} cells isolated by magnetic bead sorting demonstrated a more potent outgrowth capacity than the Sca-1-depleted population. Likewise, the Sca-1$^{+/GFP}$ outgrowths demonstrated that a highly pure population of Sca-1^{pos} cells retained outgrowth potential (100%) compared to a Sca-1-depleted population (0%). In this experiment less than 10,000 Sca-1^{pos} cells formed an outgrowth in all transplant sites, while previous studies have shown that >100,000 total cells injected is necessary to produce a mammary outgrowth in about 60% of transplant sites (Smith 1996). Thus, these experiments demonstrate that Sca-1^{pos} cells have increased outgrowth potential compared to total primary MECs.

Outgrowths from Sca-1-enriched cells developed both ductal (luminal and myoepithelial cells) and alveolar structures, suggesting that the Sca-1-enriched population either contains a mixed population of alveolar and ductal progenitors or a multipotent stem cell population (Fig. 5

and data not shown). To distinguish between these possibilities, further dilution and transplantation experiments employing the purified Sca-1 population will be required.

Relationship of Mammary Epithelial Cells

Several decades of mammary gland research have demonstrated the regenerative capacity of the mammary gland, verifying the presence and potency of mammary gland stem and progenitor cells (DeOme et al. 1959; Hoshino and Gardner 1967; Daniel et al. 1968). Stem cells in the mammary gland are expected to contribute to all mammary gland cell types including luminal, myoepithelial and alveolar. The progeny from mammary gland stem cells should not only maintain the ductal networks in a mature animal but also produce precursor cells capable of differentiation. This implies that the mammary gland consists of a mixed population of cells within a spectrum of differentiation states. Data presented here demonstrate the presence of several populations in the mammary gland with different regenerative capability and molecular markers that characterize these populations. Mammary gland Sca-1^{pos} cells make up about 20% of the total epithelial population, have increased outgrowth potential, are enriched in LRCs and MG-SP cells and are PR negative (Fig. 6A). Sca-1^{neg} cells make up about 80% of the epithelial population, have few LRCs, have decreased outgrowth potential and express differentiation markers PR and K18.

From these results, at least three cell populations in the mammary gland can be defined: PR^{pos}, Sca-1^{pos} and MG-SP cells. These results suggest that Sca-1^{pos} and MG-SP cells are not mutually exclusive and represent overlapping populations (Fig. 6A). The PR^{pos} cells, however, are excluded from the Sca-1^{pos} and presumably the MG-SP populations. The label retention technique demonstrated that the MG-SP and Sca-1^{pos} cells are proliferative during ductal

development, but are quiescent in the mature animal. Characteristics of these populations make them potential candidates for mammary epithelial stem/progenitor cells.

In the mammary gland, MG-SP cells represent a morphologically homogenous population of small cells with low cytoplasmic to nuclear ratios and decreased forward and side scatter profiles (data not shown), suggesting that these cells do not retain specialized secretory or metabolic function. The low percentage of these cells found in the mammary gland and their distinct morphological properties make MG-SP cells prime candidates for a primitive stem cell population in the mammary gland. In this model the MG-SP cells may be capable of both asymmetric (progenitor) and symmetric (self-renewal) cell division. This results in the development of a spectrum of progenitor cells that retain Sca-1 expression until further differentiation into steroid hormone-positive cell types.

The unique replicative capacity and clonal expansion of stem and progenitor cells makes these cells susceptible to transformation; it is likely that mutations occurring during stem cell proliferation may by perpetuated throughout the mammary gland. This model is supported by several lines of research: 1) in rat DMBA breast cancer models, the targets of carcinogen-induced transformation are the TEBs (the prospective site of stem cells during ductal morphogenesis), and 2) stem cell markers have been shown to be upregulated in human breast cancer (Russo et al. 1982; Dulbecco et al. 1986; Smith et al. 1990; Smith and Chepko 2001). Previous studies have found that Sca-1 is upregulated in carcinoma cell lines (including mammary lines), and higher levels of Sca-1 correlated with more aggressive, tumorigenic cell lines (Katz et al. 1994; Cohn et al. 1997; Treister et al. 1998). Preliminary data also suggest that mammary epithelial tumor cell lines yielding more aggressive tumors express higher levels of Sca-1 than less aggressive tumor cell lines (B. Welm, S. Tepera, J. Rosen, D. Medina,

unpublished observation). Therefore, given the suggestion that Sca-1^{pos} cells may be targets of transformation, it will be interesting to further characterize the status of Sca-1 expression in various breast cancer models.

In summary, these studies are the first to report methods for the isolation of functional mammary stem/progenitor cells. The availability of this population should permit the identification of additional markers that can be used to follow cell lineages during normal mammary development and the progression of breast cancer. Further, studies are in progress to compare the similarities and/or differences between these mammary progenitor cells with those isolated from other tissues.

Materials and Methods

BrdU Label Retention

Alzet minipumps #2002 (Durect Corp., Cupertino, CA) were filled with 200 µl of 60 mg/ml BrdU (Sigma #B-5002) (designed to release 0.5 µl/hr for 14 days). Three-week-old C57B6 female mice (Taconic, Germantown, NY) were anesthetized with Avertin and a minipump was implanted interscapularly into each animal. Fourteen days later, the animals were anesthetized, and the pumps were removed. This label retention experiment was conducted four times, using 19-24 mice in each experiment. Inguinal glands from mice were collected and BrdU was analyzed by immunofluorescence at week 0 (4 glands), week 9 (8 glands), and intervening weeks (2 glands each). After the 9-week chase period, lymph nodes were removed from #4 inguinal glands, and the glands were digested into mammary epithelial primary culture suspension (DeOme et al. 1959; Pullan and Streuli 1996). The cells were sorted by FACS for the MG-SP population and by magnetic column for Sca-1 expression (as described below). These sorted cells were then spun onto glass slides, fixed with cold acetone for 10 min, and washed with PBS. Label retaining cells on these slides were identified by submerging in 0.07N NaOH for 2 min, washing in PBS pH 8.5, staining with anti-BrdU-FITC (Becton Dickinson #347583) for 1 hr, and counterstaining with DAPI (Vector #H-1200). The number of $BrdU^{pos}$ cells in each of these populations was counted and graphed.

Immunofluorescence Localization

Glands were fixed in 4% paraformaldehyde for 2 hr on ice, embedded in paraffin, and cut into 5 µm sections. After deparaffinization, antigen retrieval was performed as previously described (Seagroves et al. 2000). Sections were incubated with primary antibody overnight at room

temperature in a humidity chamber, washed in PBS and incubated with secondary antibody for 1 hr at room temperature. Sections were then washed 1 hr in PBS and mounted in mounting medium containing DAPI (Vector #H-1200). Primary antibodies used were: mouse monoclonal anti-BrdU-FITC (Becton-Dickinson #347583), rabbit-anti-human PR (Dako #A0098), mouse monoclonal anti-human cytokeratin 18 (Progen #61028, clone Ks 18.04), and rabbit-anti-mouse cytokeratin 14 (Covance #PRB-155P). Secondary antibodies used were: goat-anti-rabbit-Texas Red (Molecular Probes #T6391) and goat-anti-mouse-Texas Red (Molecular Probes #T6390).

SP Sorting

All ten mammary glands were dissected out of mature virgin C57B6/129 ROSA26 mice (Jackson Labs), and the epithelial cell fraction was isolated as described previously (Pullan and Streuli 1996). Cells were plated on plastic tissue culture dishes in growth medium consisting of F12 (Gibco BRL) supplemented with insulin (5 μg/ml; Sigma), hydrocortisone (1 μg/ml; Sigma), epidermal growth factor (10 ng/ ml; Gibco BRL), penicillin/streptomycin (100 μg/ml; Gibco BRL), gentamycin (50 μg/ml; Sigma), and fetal bovine serum (10%; JRH Biosciences). After 72 hr in culture, the cells were trypsinized with 0.05% trypsin/0.02% EDTA (JRH Biosciences), washed twice in Hanks' Balanced Salt Solution (HBSS; Gibco BRL), and stained with a final concentration of 5 μg/ml Hoechst-33342 in Dulbecco's Modified Eagle's Medium (DMEM) with 2% FBS at 37°C for 90 min as described previously (Goodell et al. 1996). Sca-1 expression in the MG-SP cells was analyzed by staining the cells with anti-Sca-1-PE antibody (BD Pharmingen #553336, clone E13-161.7) for 15 min on ice following the Hoescht-33342 treatment. Analysis and sorting were performed on a triple laser MoFlow (Cytomation, Fort Collins, CO). The Hoechst dye was excited at 350 nm and its fluorescence was measured at two

wavelengths, 450/20 BP filter Blue and 675 EFLP optical filter Red, as described previously (Goodell et al. 1996).

SP Cell Injections

The inguinal glands of 21-day old RAG-/- females (Jackson Labs) were cleared of endogenous epithelium as previously described (DeOme et al. 1959). Collected SP cells were washed with HBSS, and $2.5x10^4$ or $7.5x\ 10^4$ cells were mixed with $2x10^5$ unsorted wildtype C57BL/6 cells per injection site. Cells were injected into the cleared fat pad of 21-day old mice using a 50 µl Hamilton syringe in a blind method. Cells were allowed to grow out for 6 weeks, and then the animals were bred to induce lobuloalveolar development. At day 10-16 of pregnancy, glands were surgically removed and stained for lac-Z expression as previously descrided (Rijnkels and Rosen in press).

Sca-1 Magnetic Sorting

Enrichment of Sca-1-expressing cells was achieved by sorting cells using the MACS system (Miltenyi Biotec, Sunnyvale, CA). Whole primary culture cells were incubated with biotinylated anti-Sca-1 antibody (PharMingin #553334) for 10 min on ice, washed in $DMEM^+$ (DMEM with 2% fetal bovine serum (JRH Biosciences #) and 10 mM HEPES (Sigma #), incubated with streptavidin-conjugated microbeads (Miltenyi Biotec #130-048-101) for 5 min on ice, incubated with streptavidin-PE (Molecular Probes #S-866) for 5 min on ice, washed with $DMEM^+$, and loaded onto a MACS column (Miltenyi #130-041-306). The column was set up in a Miltenyi magnet so the magnetized microbeads and all cells that adhered to them would be retained on the column. The flow through was collected as the Sca-1^{neg} fraction, and the Sca-1^{pos} cells were

eluted from the column by removing the column from the magnetic field and washing with $DMEM^{+}$. Purity of the Sca-1 enrichment and viability of the cells (by propidium iodide staining) was analyzed on a FACSCAN (Becton Dickinson, Sunnyvale, CA).

Sca-1-Bead Sorted Cell Injections

Mammary epithelial primary cultures were isolated from #4 and #5 mammary glands (without the lymph node) of C57BL6 mice (Harlan Sprague Dawley) as described above. Cells were sorted on the MACS system and Sca-1^{pos} and Sca-1^{neg} fractions were collected. Recipient C57BL6 females at 21 days of age were cleared of endogenous mammary epithelium, and 50,000 viable Sca-1^{pos} or Sca-1^{neg} cells were injected into each cleared fat pad in a blind method. After 4 weeks, a subset of the animals were bred, and at day 8-10 of pregnancy the transplanted fat pads were surgically removed and fixed in 4% paraformaldehyde for 2 hr on ice. Whole mount prepared as previously described (Williams and Daniel 1983)and images were captured using Olympus dissecting microscope and Sony video camera (#DXC-151A).

Sca-$1^{+/GFP}$ Fluorescence

Mammary glands were removed from Sca-$1^{+/GFP}$ mice, fixed in 2% paraformaldehyde for 2 hr, frozen in Tissue Freezing Medium (Triangle Biomedical Sciences #H-TFM) on dry ice, and sectioned in 30 μm sections. Sections were incubated with Texas Red-X phalloidin (Molecular Probes #T-7471) or rabbit-anti-human PR (Dako #A0098) and anti-rabbit-Texas Red (Molecular Probes #T6391) as described above except incubation times were increased to 1 hr/10 μm of section. Sections were analyzed using Zeiss 510 laser scanning confocal microscope.

Sca-1$^{+/GFP}$ Sorted Cell Injections

Mammary epithelial primary cultures were isolated from inguinal and pelvic mammary glands (without the lymph node) of C57BL6/129 Sca-1$^{+/GFP}$ mice as described above. Cells were sorted on a Beckman Coulter Altra FACS machine with an argon laser tuned to 488 nm. GFP fluorescence was measured at 525 nm BP filter, GFP^{pos} and GFP^{neg} fractions were collected in DMEM with 2% FBS, and data was analyzed using Expo 32 software. Ten thousand viable Sca-1^{pos} or Sca-1^{neg} cells were injected into each cleared fat pad of Rag$^{-/-}$ females as described above. After six weeks, the transplanted fat pads were surgically removed and whole mounts were prepared as described above.

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Figure Legends

Figure 1. Long Term BrdU Label Retention

(A) A schematic representation of the experimental design. Three-week old female mice received a continuous dose of BrdU for two weeks via a subcutaneously implanted pump. Upon removal of the pump, mammary gland biopsies were taken each week during the 9-week chase period. After 9 weeks, mammary glands were analyzed for label-retaining cells (LRCs), a small population of quiescent epithelial cells. (B-E) Immunofluorescence analysis of BrdU-labeled cells at week 0 (B,D) and week 9 (C,E). Luminal epithelium was extensively labeled with BrdU at week 0 (B), but only about 5% of luminal epithelium retain this label in week 9 (C). Double immunofluorescence analysis (BrdU-FITC and PR-Texas Red) revealed colocalization of $BrdU^{pos}$ and PR^{pos} cells at week 0 (D), but very little colocalization by week 9 (E, arrows). Quantitation of PR^{pos} and $BrdU^{pos}$ cells throughout the chase period (F) showed constant numbers of PR^{pos} cells, decreasing numbers of $BrdU^{pos}$ cells, and decreasing colocalization of PR and BrdU. (G-J) Colocalization of LRCs with cytokeratin 14- and cytokeratin 18-expressing cells after 9-week chase (BrdU-FITC (G), K14-Texas Red and K18-Texas Red (H), BrdU and K14/18 (I), and DAPI (J)). Two distinct populations of LRCs expressed K14/K18 (arrowheads) and did not express these markers of differentiation (arrows), suggesting LRCs represent a spectrum of differentiated cells. Scale bars represent 5 μm (B-E) and 1 μm (G-J).

Figure 2. Mammary Gland Contains a Pluripotent Population of MG-SP Cells

FACS analysis of Hoechst-33342 exclusion reveals a population of cells which effluxes Hoechst dye (A). These cells represent approximately 3% of total mammary epithelial cells and were termed MG-SP cells. This MG-SP population was enriched for expression of Sca-1 (C) when compared to Sca-1 expression in total MECs (B). $Sca\text{-}1^{pos}$ cells isolated from total MEC culture do not express the

peripheral blood marker CD-45 (D). When MG-SP cells were isolated from glands following BrdU incorporation and a 9-week chase, the MG-SP population was enriched for LRCs four-fold compared to the non-MG-SP cells (E). (F and G) Competitive recombination experiments using MG-SP cells isolated from ROSA26 mammary glands. MG-SP cells, injected into cleared fat pads of host mice, reconstituted an epithelial outgrowth. X-gal staining (indicating the outgrowth originated from ROSA26 MG-SP cells) revealed two clonal outgrowths (F, arrowheads). Higher magnification (G) illustrates X-gal staining in both ductal (arrowheads) and alveolar (arrows) cell types, suggesting MG-SP cells maintained the ability to differentiate into multiple cell lineages in the mammary gland. Scale bars represent 1 mm.

Figure 3. Expression of Sca-1 in the Mammary Gland

Mammary glands were removed from Sca-$1^{+/GFP}$ mice and analyzed for GFP localization by immunofluorescence (GFP and phalloidin-Texas Red (A); GFP and PR-Texas Red (B)). A cross-section of ductal epithelium shows GFP expression detected sporadically in luminal epithelial cells (A). The abundance of GFP-expressing cells correlates with about 20% Sca-1^{pos} cells detected by FACS analysis (Fig. 2B and Fig. 4A). Three-dimensional rotation of this confocal image reveals GFP-expressing cells in contact with the lumen and others located basally. Additionally, GFP expression does not colocalize with PR expression in luminal epithelial cells (B). Scale bars indicate 1 μm.

Figure 4. Enriching Sca-1^{pos} Cells by Magnetic Sorting

Sca-1^{pos} cells were isolated from total mammary epithelial cells by immunosorting using biotinylated anti-Sca-1 antibodies and streptavidin-conjugated microbeads. The results of a single round of enrichment are illustrated in (A-C): whole mammary epithelial cell fraction (A), Sca-1-depleted fraction

(B), and Sca-1-enriched fraction (C). When these fractions were analyzed for LRCs, the Sca-1-enriched fraction contained a two-fold enrichment of LRCs compared to the Sca-1-depleted fraction (D).

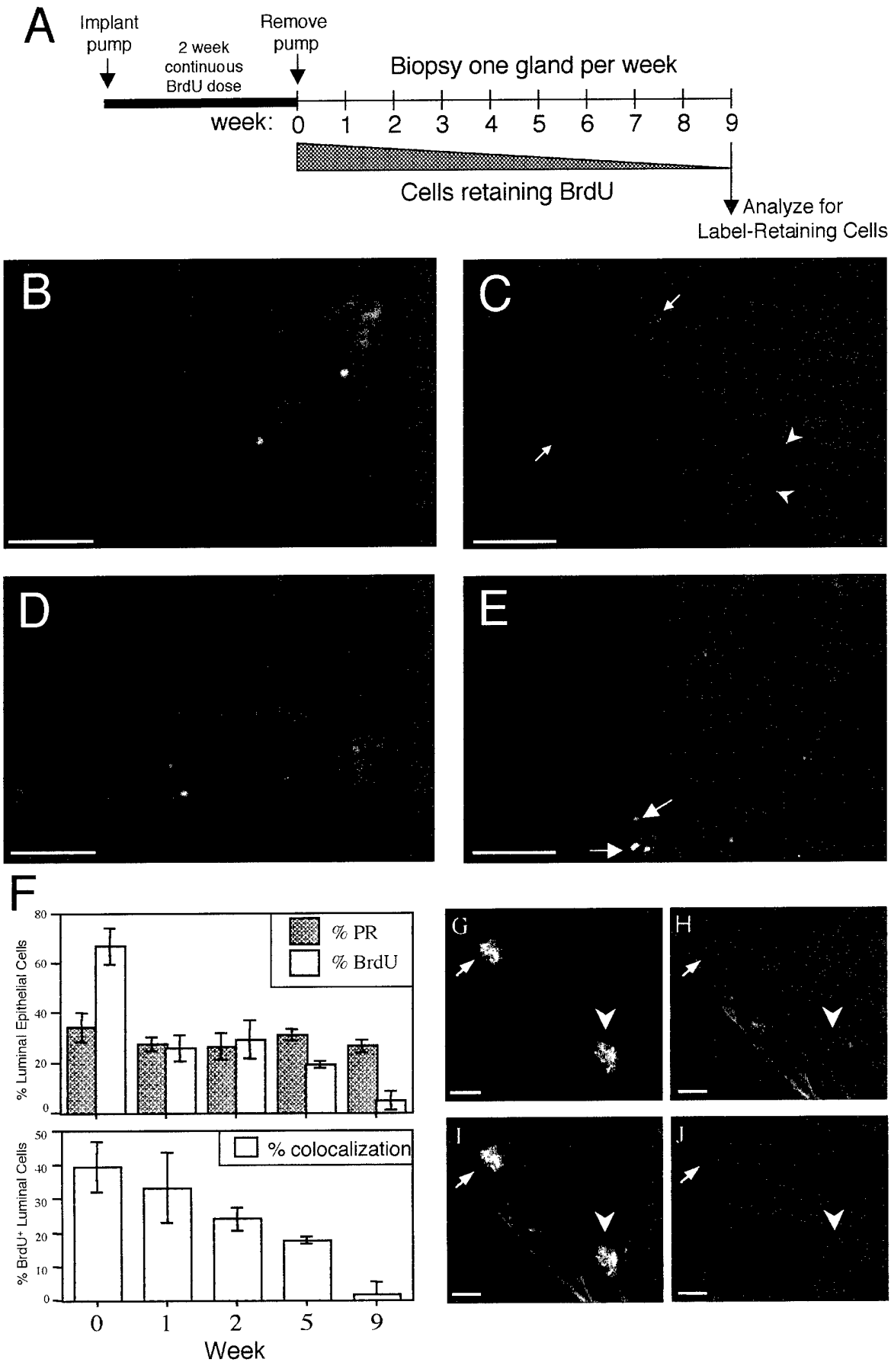
Figure 5. Outgrowth Potential of Sca-1-Sorted MECs

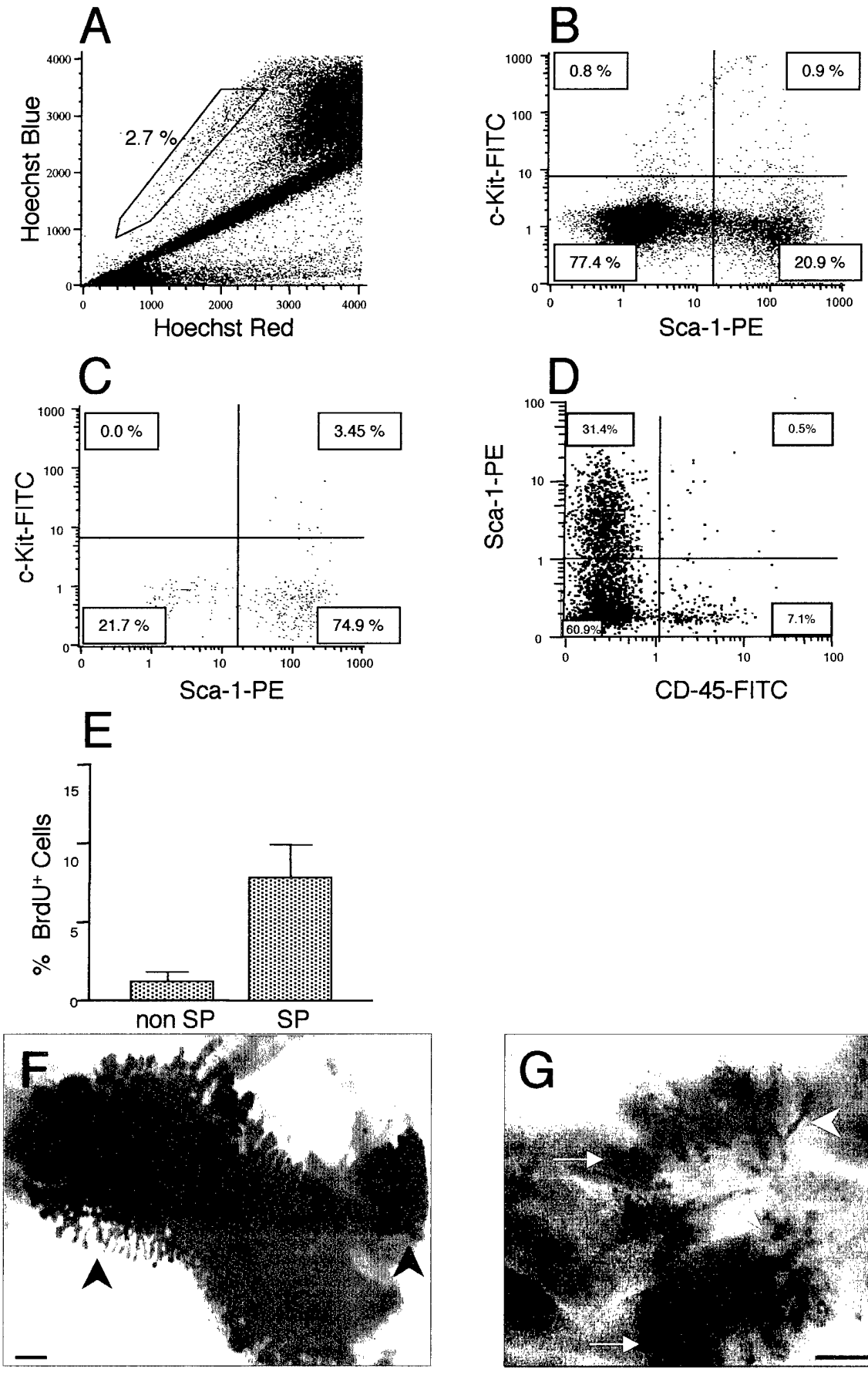
Using two methods of sorting, mammary epithelial cells were enriched for or depleted of Sca-1-expressing cells and were injected into cleared fat pads in a standard mammary reconstitution assay. Outgrowths were harvested after 6 weeks and the epithelium in the whole mounts was visualized by hematoxylin (Fig. 5A,B,C). Injected cells either formed no epithelial structure (A), a partial but underdeveloped epithelial outgrowth (B), or a well developed ductal network (C). When cells were sorted using magnetic bead enrichment, all injections of Sca-1-enriched cells resulted in epithelial outgrowths, but less than 50% of Sca-1-depleted injections formed an outgrowth. When Sca-$1^{+/GFP}$ cells were sorted for GFP fluorescence, 100% of Sca-1-enriched cells grew out, while none of the Sca-1-depleted cells formed a mammary outgrowth. (D-F) Normal ductal and alveolar development was detected in Sca-1-enriched outgrowths. Whole mounts (D) and H&E stained sections (E, F) of Sca-1^{pos} outgrowths showed luminal, myoepithelial (F, arrowheads) and TEBs (E) with normal cap (arrowhead) and body (arrow) cell layers. Alveolar buds were observed in Sca-1^{pos} outgrowths of early pregnant mice (D,F, arrows).

Figure 6. Model of MEC Progenitors

Mammary epithelium can be classified into several distinct cell populations including MG-SP, Sca-1^{pos}, and PR^{pos} cells. In this model, these populations may represent various stages of epithelial differentiation. LRCs are found primarily in the Sca-1 and MG-SP populations suggesting that these groups contain within them a quiescent sub-population. The MG-SP

population may represent the most primitive and least differentiated subclass and may contain the multipotent stem cell population.


A
Implant pump
2 week continuous BrdU dose
Remove pump
Biopsy one gland per week
week: 0 1 2 3 4 5 6 7 8 9
Cells retaining BrdU
Analyze for Label-Retaining Cells
B
C
D
E
F
% PR
% BrdU
% Luminal Epithelial Cells
% colocalization
% BrdU+ Luminal Cells
Week
G
H
I
J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Hoechst Blue
Hoechst Red
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B
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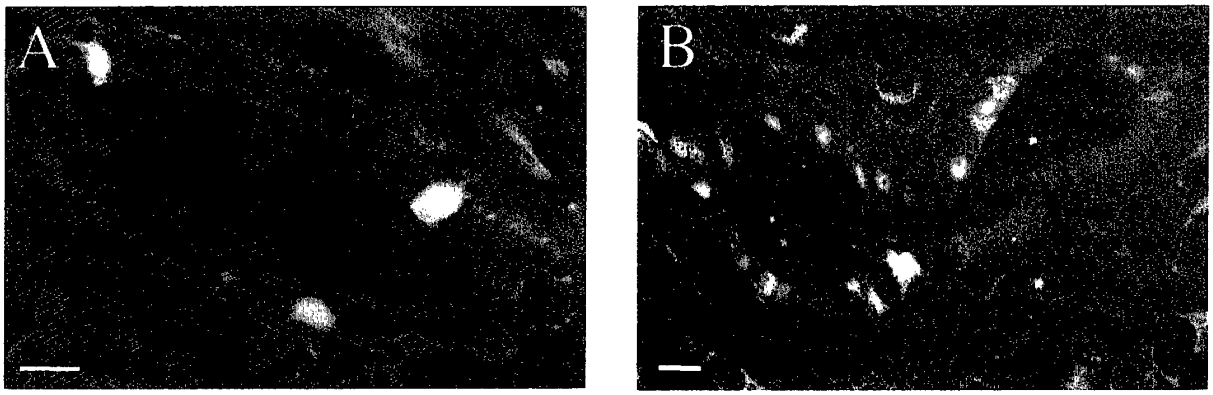
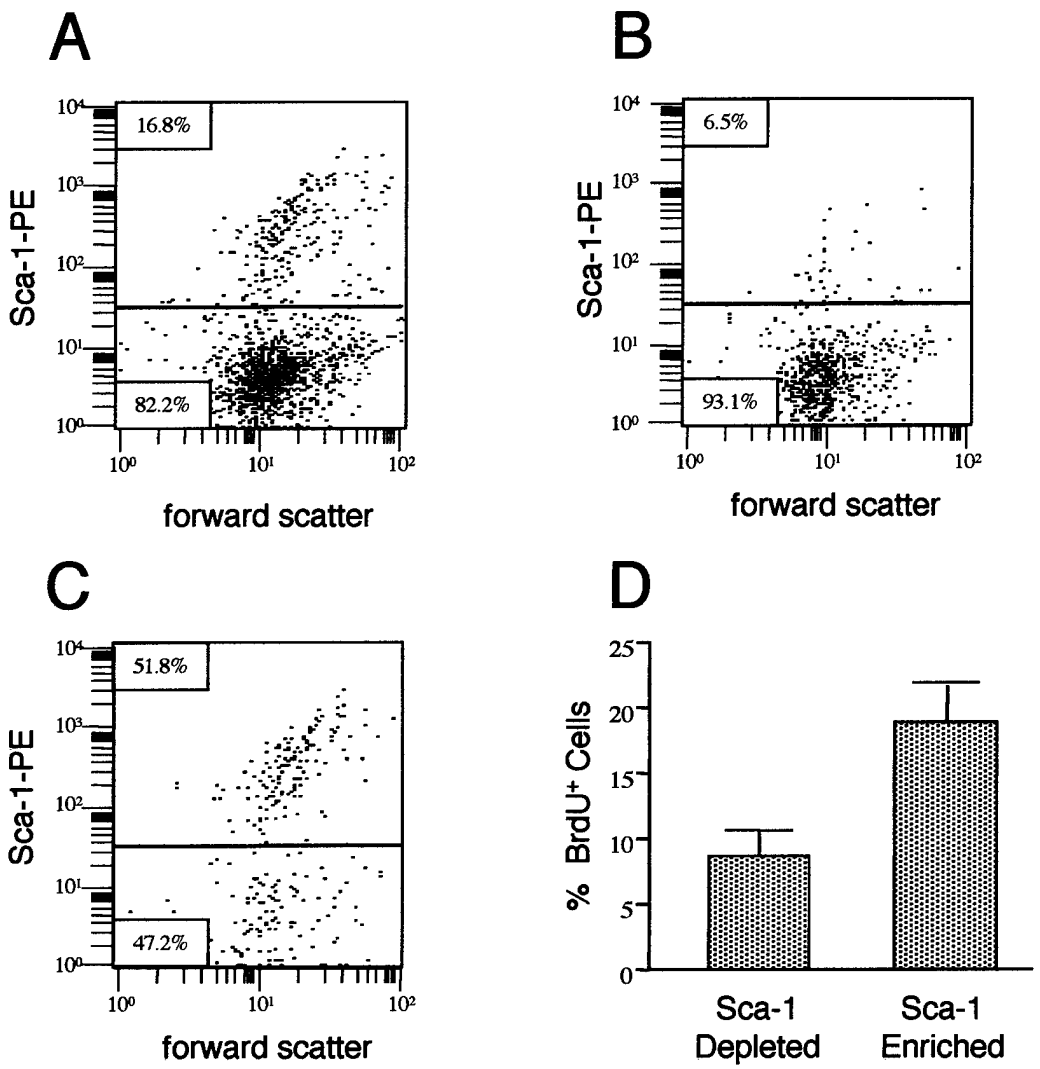
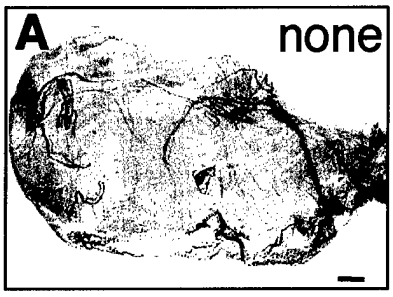
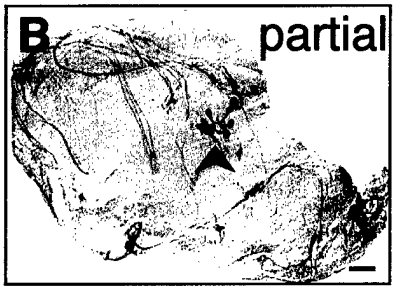
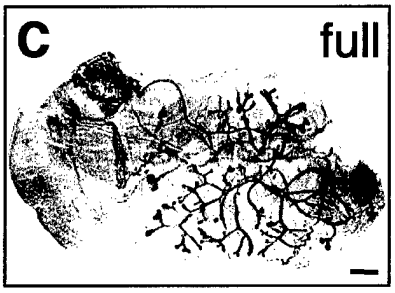
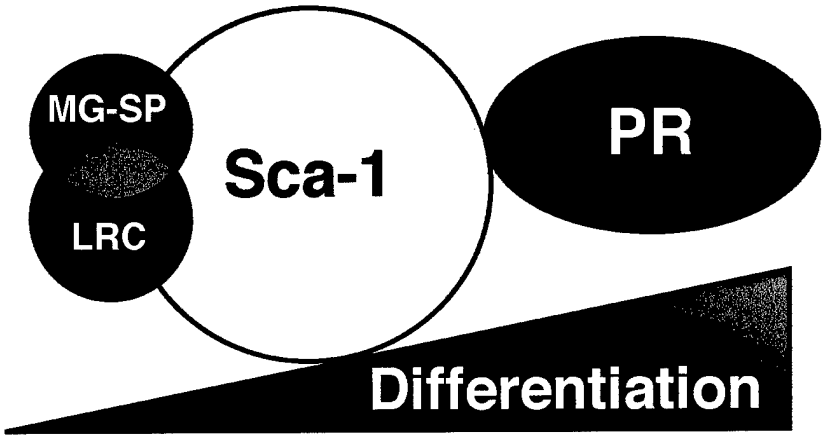
Sca-1-PE
0.8 %
0.9 %
77.4 %
20.9 %
C
c-Kit-FITC
Sca-1-PE
0.0 %
3.45 %
21.7 %
74.9 %
D
Sca-1-PE
CD-45-FITC
31.4%
0.5%
60.9%
7.1%
E
% BrdU+ Cells
non SP
SP
F
G


A
B


A
16.8%
82.2%
Sca-1-PE
forward scatter
B
6.5%
93.1%
Sca-1-PE
forward scatter
C
51.8%
47.2%
Sca-1-PE
forward scatter
D
% BrdU+ Cells
Sca-1 Depleted
Sca-1 Enriched

	Magnetic Bead Enrichment		Sca-1$^{+/GFP}$	
	Sca-1^{pos}	Sca-1^{neg}	Sca-1^{pos}	Sca-1^{neg}
	0/6	4/6	0/6	6/6
	2/6	1/6	3/6	0/6
	4/6	1/6	3/6	0/6
Total outgrowths:	6/6	2/6	6/6	0/6


MG-SP
LRC
Sca-1
PR
Differentiation